British Journal of Cancer (2009) 100, 370 – 375

www.bjcancer.com

LKB1/KRAS mutant lung cancers constitute a genetic subset of NSCLC with increased sensitivity to MAPK and mTOR signalling inhibition

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LKB1/STK11 is a multitasking tumour suppressor kinase. Germline inactivating mutations of the gene are responsible for the Peutz-Jeghers hereditary cancer syndrome. It is also somatically inactivated in approximately 30% of non-small-cell lung cancer (NSCLC). Here, we report that *LKB1/KRAS* mutant NSCLC cell lines are sensitive to the MEK inhibitor CI-1040 shown by a dose-dependent reduction in proliferation rate, whereas LKB1 and KRAS mutations alone do not confer similar sensitivity. We show that this subset of NSCLC is also sensitised to the mTOR inhibitor rapamycin. Importantly, the data suggest that *LKB1/KRAS* mutant NSCLCs are a genetically and functionally distinct subset and further suggest that this subset of lung cancers might afford an opportunity for exploitation of anti-MAPK/mTOR-targeted therapies.
British Journal of Cancer (2009) **100,** 370 – 375. doi:10.1038/sj.bjc.6604886 www.bjcancer.com

Keywords: LKB1; KRAS; NSCLC; CI-1040; MEK

LKB1/STK11 is a serine threonine kinase known to be involved in several cellular processes including, signal transduction, energy sensing and cell polarity (Spicer and Ashworth, 2004). Germline inactivating mutations of *LKB1* are known to cause Peutz-Jeghers syndrome, a hereditary condition, which results in the development of benign (hamartomatous) polyps in gastrointestinal tract, mucocutaneous pigmentation (Hemminki *et al*, 1998; Mehenni *et al*, 1998) and a predisposition to developing cancers in a variety of tissues; colon, small intestine, breast, ovary, pancreas and lung (Giardiello *et al*, 1987). Approximately 30% of somatic lung adenocarcinomas harbour an inactivating mutation in *LKB1* (Sanchez-Cespedes *et al*, 2002), and a recent study lists *LKB1* as one of the four most frequently mutated genes in lung adenocarcinoma (Ding *et al*, 2008).

In mammalian cells, LKB1 exists in a complex with STE20-related adaptor (STRAD) and mouse protein 25 (MO25; Boudeau *et al*, 2003). It is a member of the calcium/calmodulin regulatory kinase-like family and is thought to phosphorylate and activate at least 13 kinases, including AMPK (Lizcano *et al*, 2004; Jaleel *et al*, 2005). AMP-activated protein kinase (AMPK) is a regulator of cellular energy metabolism, and the main function of the pathway is to restore cellular energy levels and is switched on in low ATP, high AMP conditions, caused by processes, such as hypoxia and cellular stress (Hardie *et al*, 2003). Activated AMPK phosphorylates TSC2 activating the TSC1-2 complex, which inhibits RAS homologue enriched in brain (RHEB), a small GTP-binding protein and prevents the activation of mammalian target of rapamycin (mTOR; Garami *et al*, 2003). The mammalian target of rapamycin kinase plays a central role in regulating protein synthesis and control of cell growth (cell size and mass) (Schmelzle and Hall, 2000; Fingar *et al*, 2002). Loss of LKB1, therefore, leads to a failure to inhibit RHEB and loss of suppression of mTOR signalling, leading to increases in cell size and mass (Corradetti *et al*, 2004; Shaw *et al*, 2004).

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Received 13 October 2008; revised 4 December 2008; accepted 17 December 2008

MATERIALS AND METHODS

Cell culture

Cell lines of a known genetic background (Table 1) were grown in RPMI (Invitrogen, Life Technologies Inc., Carlsbad, CA, USA) apart from CAL12T, which was grown in DMEM (Invitrogen) supplemented with 10% FBS and 1% PSG.

Statistical analysis

Statistical analysis of the co-occurrence of *LKB1* and *ras-MAPK* pathway mutations in 87 NSCLC cell lines was carried out by the Fisher's exact test.

CI-1040/rapamycin treatment and proliferation assay

Cells were seeded in six replicates to 48-well plates. After 24 h, this was replaced with media containing 0, 0.1, 0.5, 1, 5, 10 μM CI-1040 or 0, 0.1, 0.5, 1, 5, 10, 25, 50, 75, 100 nM rapamycin (Sigma-Aldrich Co, St Louis, MO, USA). After 72 h, the proliferation rate was measure using the CyQuant proliferation assay kit (Invitrogen) according to the manufacturers instructions. Statistical analysis was carried out using two-tailed unpaired *t*-tests.

Table 1 Mutation status of cell lines used in this study

		LKB1	BRAF	KRAS	PIK3CA	P53	CDNKA	EGFR
Cell line	**Histology**	**Mutation**	**Mutation**	**Mutation**	**Mutation**	**Mutation**	**Mutation**	**Mutation**
SKMEL28	Malignant melanoma		c.1799T>A p.V600E			c.434_435TG>GT p.L145R		c.2257C>T p.P753S
CAL-12T	NSCLC nos.	No protein[a]	c.1397G>T p.G466V			c.404G>T p.C135F	c.172C>T p.R58*	
A549	NSCLC nos.	c.109C>T p.Q37*		c.34G>A p.G12S			c.1_471del471 p.M1_*157del	
NCI-H1734	NSCLC adenocarcinoma	c.152_153insCT p.M51fs*14		c.37G>T p.G13C				
NCI-H460	NSCLC large cell carcinoma	c.109C>T p.Q37*		c.183A>T p.Q61H	c.1633G>A p.E545K		c.1_457del457 p.?	
NCI-H2030	NSCLC Adenocarcinoma	c.949G>T p.E317*		c.34G>T p.G12C		c.785G>T p.G262V		
NCI-H1563	NSCLC adenocarcinoma	c.816C>A p.Y272*					c.1_471del471 p.M1_*157del	
NCI-H2009	NSCLC adenocarcinoma			c.35G>C p.G12A		c.818G>T p.R273L		
NCI-H1975	NSCLC adenocarcinoma				c.353G>A p.G118D	c.818G>A p.R273H	c.205G>T p.E69*	c.2573T>G p.L858R
NCI-H1838	NSCLC adenocarcinoma					c.818G>T p.R273L	c.1_471del471 p.M1_*157del	

Mutation status of oncogenes and tumour suppressors known to be commonly mutated in NSCLC. Mutation data taken from COSMIC (http://www.sanger.ac.uk/genetics/CGP/CellLines/). [a]No mutation has been found in this sample by sequencing; however, immunoblot analysis revealed no protein present (data not shown); NSCLC nos. = NSCLC not otherwise specified.

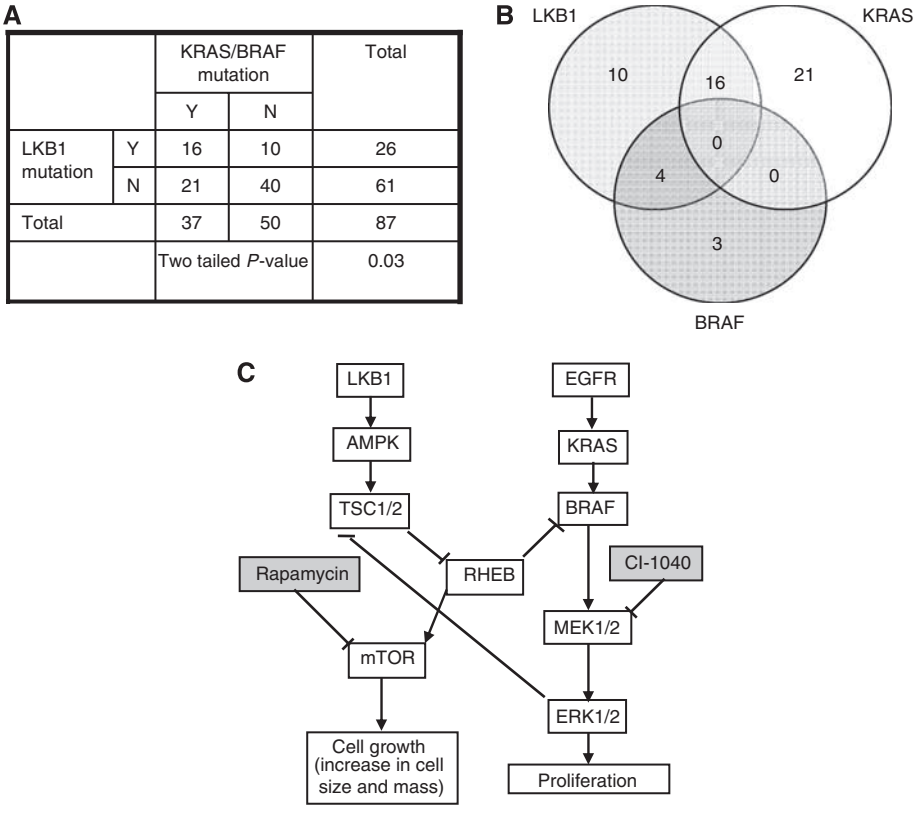

Figure 1 Statistical and biological significance of LKB1 mutations and RAS-MAPK pathway mutations. (**A**) Statistical analysis of 87 lung cancer cell lines by the Fisher's exact test. (**B**) Venn diagram showing the overlap of LKB1, KRAS and BRAF mutations. (**C**) Snapshot of cross-talk between LKB1 and RAS-MAPK signalling pathways compiled by the analysis of literature (for references, see main text).

Immunoblotting

Cells were seeded to 6-well plates; 24 h later, this was replaced with media containing 0, 0.1, 0.5, 1, 5, 10 μM CI-1040 or 0, 1, 10, 50, 100, 200 nM rapamycin. Then 8 and 24 h after the addition of CI-1040 and 24 h after the addition of rapamycin, protein was harvested in RIPA buffer containing protease (Sigma) and phosphatase (Roche Applied Science, UK) inhibitors. Samples were mixed with pre-

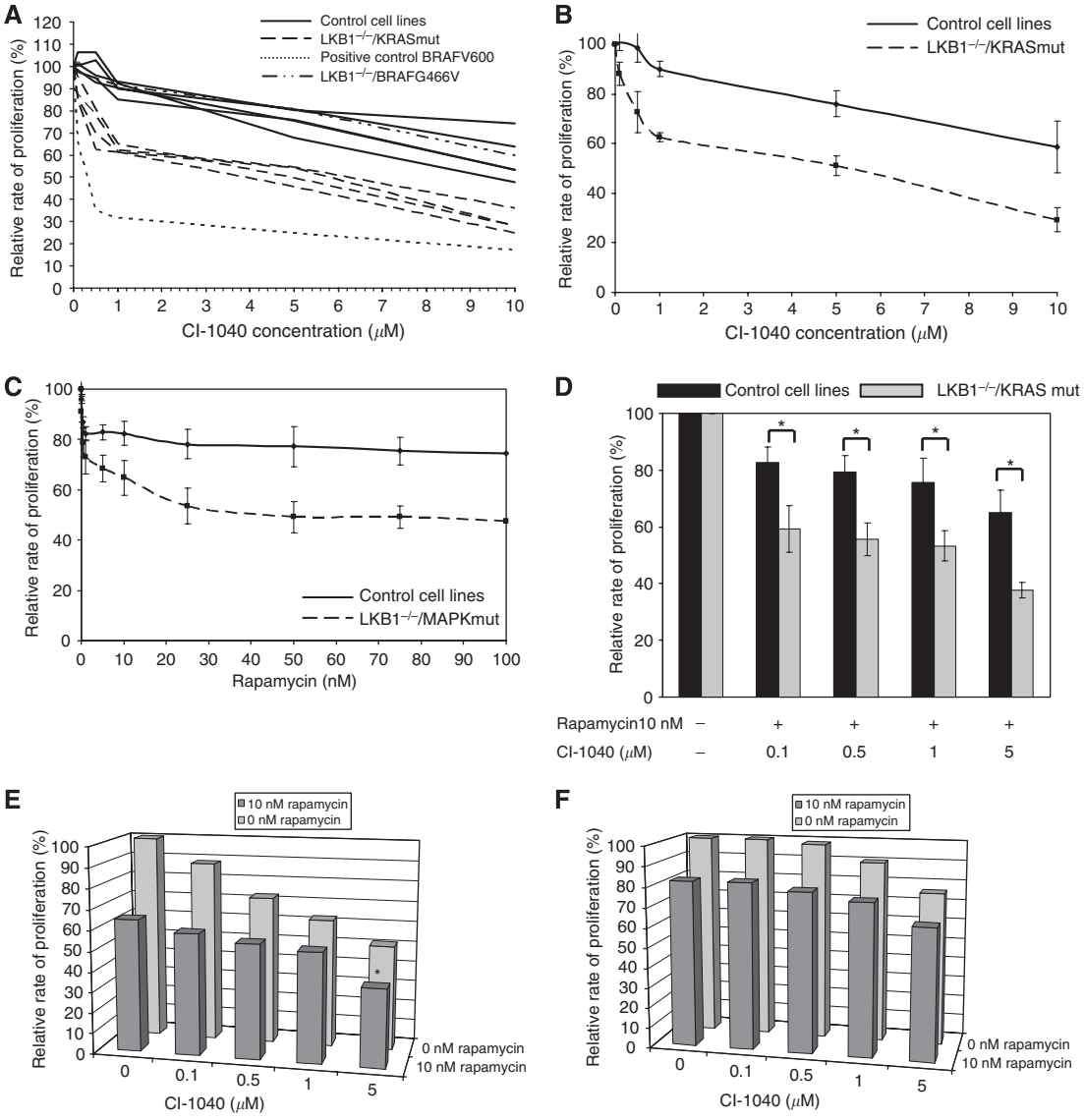

Figure 2 Cells with inactivated *LKB1* and activated *KRAS* are more sensitive to the MEK inhibitor CI-1040 and the mTOR inhibitor rapamycin; however, dual inhibition is neither additive nor synergistic. Cell lines tested: NCI-H460 (*LKB1*$^{-/-}$*/KRASmut*), A549 (*LKB1*$^{-/-}$*/KRASmut*), NCI-H1734 (*LKB1*$^{-/-}$*/KRASmut*), NCI-H2030 (*LKB1*$^{-/-}$*/KRASmut),* collectively labelled as *LKB1*$^{-/-}$*/KRASmut* in the figure; NCI-H1838 (wt), NCI-H1975 (wt), NCI-H2009 (*KRASmut*), NCI-H1563 (*LKB1*$^{-/-}$), collectively labelled as control cell lines; CAL12T (*LKB1*$^{-/-}$*/BRAFmut*), and SKMEL28 (positive control, *BRAF V600*) (**A**) Cells were seeded in six replicates to 48-well plates. After 24 h, this was replaced with a medium containing 0, 0.1, 0.5, 1, 5 or 10 μM CI-1040. After 72 h, proliferation rate was determined using CyQuant proliferation assay (Invitrogen) according to the manufacturer's instructions. The results shown here are from two independent experiments. Proliferation rates were measured relative to the untreated control. (**B**) Values from the two clusters shown in panel A were averaged to calculate the statistical significance between the clusters, values shown ± s.d between the cell lines within the cluster, $n \geqslant 4$ for each cluster. Two-tailed unpaired *t*-test gave a *P*-value of <0.001 for all CI-1040 concentrations. (**C**) The same protocol as in panel A was followed; however, after 24 h, this was replaced with a medium containing 0, 0.1, 0.5, 1, 10, 25, 50, 75 and 100 nM rapamycin. The results shown here are again from two independent experiments. Proliferation rates were measured relative to the untreated control. Owing to the similar nature of the results of the CI-1040 experiment, the cell lines were clustered according to their mutation status (*LKB1*$^{-/-}$*/KRASmut*) or control cells (*LKB1*$^{-/-}$*, WT and KRAS mut*). Unpaired two-tailed *t*-tests carried out to determine statistical significance; $P<0.05$ for concentrations of 10 nM and upwards, $n=3$ for each cluster means ± s.d. (**D**) Dual inhibition experiments were carried out using 10 nM rapamycin and a range of CI-1040 concentrations in the same format as single-agent experiments, cell lines were again grouped by their mutation status and statistical significance calculated using two-tailed unpaired *t*-tests, $P \leqslant 0.01$; results are from two independent experiments each with six replicates, mean ± s.d. (**E**) Comparison of dual-agent treatment to most potent single-agent treatment to determine whether the agents are additive/synergistic in the *LKB1*$^{-/-}$*/KRASmut* group. Statistical significance determined using unpaired two-tailed *t*-tests between single-agent treatment group and each dual-treatment group. The only significant value marked $*P=0.005$, $n=3$, mean ± s.d. (**F**) Comparison of dual-agent treatment to most potent single-agent treatment in control cell lines to determine whether the agents are additive/synergistic in the control group. No statistically significant values were found, $n=3$, means ± s.d.

made sample buffer and reducing agent (NUPAGE) and transferred to a PVDF membrane (Invitrogen) by western blotting. Membranes were probed with rabbit anti-ERK, anti-phospho ERK (Cell Signalling, 1/1000), anti-cyclin D1 (Santa Cruz, 1/500), p70S6K and phosphop70S6K (thr389) (Cell Signalling, 1/1000), followed by secondary antibodies (Cell Signalling, 1/2500). The bands were displayed using enhanced chemiluminescence method (Pierce Biotechnology Inc., Rockford, IL, USA).

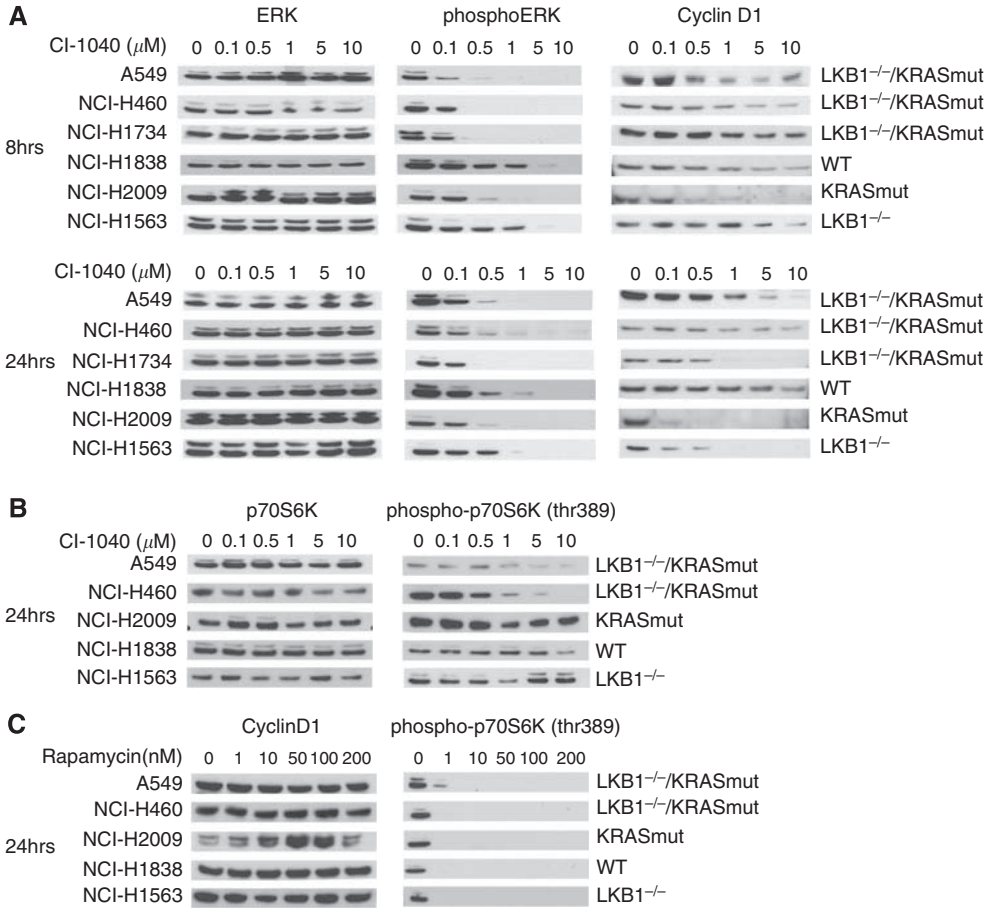

Figure 3 The sensitivity to the MEK inhibitor CI-1040 and the mTOR inhibitor rapamycin are not due to downstream effects on cyclin D1, but in the case of MEK inhibition are due to downstream effects on phospho-P70S6K (thr389). (**A**) To determine the effect of MEK inhibition on MAPK downstream pathway components cells were seeded to 6- well plates, and the following day, it was replaced with media containing 0, 0.1, 0.5, 1, 5, 10 μM CI-1040. After 8 and 24 h, the addition of CI-1040 protein was harvested in RIPA buffer containing protease (Sigma) and phosphatase (Roche) inhibitors. Samples were mixed with premade sample buffer and reducing agent (NUPAGE), and heated at 95°C for 5 mins. In all, 10 ug of protein was run on a 4–12% precast gel (NUPAGE), and transferred to a PVDF membrane (Invitrogen) by western blotting. Membranes were probed with either rabbit anti-ERK, antiphospho ERK (Cell Signalling, 1/1000), anticyclin D1 (santa-cruz, 1/500) antibodies, followed by secondary antibodies (Cell Signalling, 1/2500). The bands on the membrane were displayed using enhanced chemiluminescence method (Pierce). (**B** and **C**) Using the same immunoblotting protocol as above, mTOR downstream pathway components were assessed after treatment with CI-1040 or rapamycin. Total p70S6K and phospho-p70S6K (thr389; Cell signalling, 1/1000) protein levels were analysed in 0, 0.1, 0.5, 1, 5, 10 μM CI-1040- treated cell lines. Phospho-p70S6K (thr389) and cyclin D1 protein levels were analysed in cell lines treated with 0, 1, 10, 50, 100 and 200 nM rapamycin.

RESULTS

Resequencing of known cancer genes in a series of 87 NSCLC cell lines (http://www.sanger.ac.uk/genetics/CGP/CellLines/) found significant association of *LKB1* inactivating mutations with *KRAS* mutations ($P = 0.03$). This association has recently been confirmed in an independent study (Matsumoto *et al*, 2007). Association of *LKB1* inactivation clustering with *non-V600 BRAF* mutations (Figures 1A and B) was also observed. LKB1 and RAS/RAF/MEK (MAPK) signalling pathways are linked through RHEB, which when active, activates mTOR and inhibits wild-type BRAF, but not the mutated form (Im *et al*, 2002; Garami *et al*, 2003; Karbowniczek *et al*, 2004). It was recently shown that the inhibition of RAF1 (cRAF) activity by RHEB prevents heterodimerisation of BRAF and RAF1 (Karbowniczek *et al*, 2006). *LKB1* mutations in NSCLC may, therefore, have a general requirement for an activation of the MAPK cascade to overcome suppression through RHEB inhibition. This interdependence suggests that the inhibition of MAPK signalling may constitute a potential opportunity for therapeutic intervention in this genetic subset of NSCLC (Figure 1C).

To further explore this potential, NSCLC lines of known genetic backgrounds (Table 1) were treated with the MEK inhibitor CI-1040. Figure 2A shows that the *LKB1/KRAS* mutant cell lines have a uniform enhanced sensitivity to CI-1040 when compared with wild-type cell lines, *LKB1* mutant lines or *KRAS* mutant lines (labelled control cell lines in Figure 2B). Interestingly, the *LKB1/BRAFG466V* mutant cell line (CAL12T) is insensitive to CI-1040 and falls in the top cluster. The mean relative proliferation rate calculated for *LKB1/KRAS* mutant cell lines, and compared with the control cell line cluster was statistically significant ($P < 0.001$ at all CI-1040 concentrations > 0; Figure 2B). *LKB1/KRAS* mutant cell lines have a mean IC_{50} value of 5 μM compared with the control cell lines, which have a mean $IC_{50} > 10$ μM. We next investigated the importance of mTOR signalling in the genetic subtypes of lung cancers under study. Figure 2C shows that inhibiting mTOR using rapamycin had a more pronounced affect on proliferation in *LKB1/KRAS* mutant cell lines; in this case, the sensitive cluster also included the *LKB1/BRAF* mutant cell line CAL12T. The IC_{50} of the *LKB1/MAPK* mutant cluster was significantly different from the control cluster (40 nM *vs* >100 nM, $P \leqslant 0.04$). Dual inhibition of MEK and mTOR (Figure 2D) leads to a statistically significant

decrease the proliferation rate in the *LKB1/KRAS* mutant cluster *versus* the control cell lines (*P*-value: $\leqslant 0.01$). This effect of dual inhibition in general did not reach statistical significance for additivity nor being synergistic in either cluster (Figures 2E and F), although at the highest concentrations (5 μM CI-1040 with 10 nM rapamycin) in the *LKB1/KRAS* mutant cluster, the data were consistent with an additive model. However, this may be due to the combined toxic effects of higher drug concentrations.

To confirm that MEK inhibition was being achieved in the cells, we carried out immunoblot analysis of ERK, phosphorylated ERK and cyclin D1 levels at 8 and 24 h following CI-1040 treatment (Figure 3A). In all cell lines, levels of phosphorylated ERK decreased with increasing CI-1040 concentration by 8 h; however, wild-type cell lines for both genes or *LKB1* mutant required higher concentrations of CI-1040 to prevent phosphorylation of ERK. The effect of MEK inhibition on cyclin D1 levels did not appear to correlate with genetic status, and interestingly, the *KRAS* mutant cell line NCI-H2009 showed a similar decrease in phosphorylated ERK, and perhaps the greatest decrease in cyclin D1 levels, despite the inhibitor having little effect on proliferation. Altogether, these data show that the effects of MEK inhibition on phospho-ERK are driven by the presence or absence of a *KRAS* mutation and are independent of *LKB1* mutation status, whereas the proliferation effects are related to *LKB1/KRAS* combined mutation status. As there was no correlation with cyclin D1 levels and enhanced sensitivity to MEK inhibition, we carried out immunoblot analysis of p70S6K and phospho-p70S6K (thr-389) levels; phosphorylation of this residue is critical for kinase function (Pullen and Thomas, 1997). Figure 3B shows that CI-1040 treatment had no effect on total p70S6K protein levels; however, a decrease was observed in phospho-p70S6K (thr-389) levels, specifically in LKB1/KRAS mutant cell lines. This decrease in phosphorylation correlated well with the observed IC_{50} for this genetic subset. Figure 3c shows that rapamycin treatment had no effect on cyclin D1 protein levels, but had a potent effect on phospho-p70S6K (thr-389) levels in all cell lines regardless of the mutation status.

DISCUSSION

Here, we report that *LKB1* inactivation and *KRAS* activation in non-small-cell lung cancer denotes a functionally distinct set of lung cancer, which display sensitivity to the single-agent treatment with the MEK inhibitor CI-1040 or rapamycin. It has been previously shown in melanomas that treatment with CI-1040 caused a dose-dependent reduction in phospho-ERK and cyclin D1 protein levels in *BRAFV600E* mutant cell lines, but not in *NRAS* mutant melanomas (Solit *et al*, 2006). Our results, while showing dose-dependent reduction of phospho-ERK in *KRAS* mutant NSCLC cell lines, show inhibition of proliferation only in the subset with both *KRAS* and *LKB1* mutations, highlighting the importance of the cross-talk in these pathways and supporting the genetic data that the co-occurrence of these two mutations is non-random. Dose-dependent decrease in phospho-ERK and reduction in proliferation rate did not result in a corresponding decrease in cyclin D1 levels, suggesting that in this subset of NSCLC, the downstream effecter pathways may be different to *BRAFV600E* melanomas. The effects of reduced proliferation in this genetic subset may be due to the reduction in the activity of p70S6K, which is downstream of both mTOR and ERK1/2, shown by the specific reduction of phosphorylation of thr-389 P70S6K in LKB1/KRAS cell lines following MEK inhibition. These data further highlight the importance of cross-talk between these pathways in this genetic subset. Interestingly, CAL12T, the *LKB1/BRAF* mutant cell line is insensitive to CI-1040 yet sensitive rapamycin, suggesting an additional, smaller genetic subset in NSCLC and possibly highlighting the difference of the non-*V600 BRAF* mutations found in NSCLC (Brose *et al*, 2002). Despite being sensitised to CI-1040 and rapamycin, dual-agent treatment did not have demonstrably additive or synergistic effects in *LKB1/KRAS* cell lines, suggesting the possible redundancy in the pathways. The lack of additivity may be explained by the observation that rapamycin potently inhibits p70S6K phosphorylation at thr-389, therefore precluding any additional effect of CI-1040 on the p70S6K activity, further confirming redundancy of the pathways in this genetic subset of NSCLC.

Mutation status in cancers has been shown to predict response to targeted therapies, as exemplified by the efficacy of EGFR inhibitors in EGFR mutant lung cancer (Lynch *et al*, 2004; Paez *et al*, 2004). Data presented here suggest that *LKB1/KRAS* mutated tumours are a genetic and functionally distinct subset of NSCLC. Further, these data suggest that investigation of this subset of lung cancers with respect to newer generation inhibitors of MAPK and mTOR signalling pathways may provide a new opportunity for investigation of targeted therapeutics in this common adult malignancy.

ACKNOWLEDGEMENTS

This study was supported by the Wellcome Trust and Glaxo Smith Kline. We thank Richard Marais for providing us with CI-1040 and Peter Campbell for critically reading the manuscript.

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